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Dual role of cAMP during *Dictyostelium* development

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Abstract. cAMP plays an essential role during *Dictyostelium* development both outside and inside the cell. Membrane-bound receptors and adenylyl cyclase are responsible for sensing and producing extracellular cAMP, whereas a phosphodiesterase is responsible for maintaining a low basal level. The molecular events underlying this type of hormone like signalling, which are now beginning to be deciphered, will be presented, in the light of cAMP analogue studies. The importance of intracellular cAMP for cell differentiation has been demonstrated by the central role of the cAMP dependent protein kinase. Mutants as well as strains obtained by reverse genetics will be reviewed which lead to our current understanding of the role of intracelluar cAMP in the differentiation of both stalk and spore cells.

Key words. Cyclic AMP; receptors; adenylyl cyclase; phosphodiesterase; protein kinase; cAMP analogs.

Introduction

Dictyostelium fruiting body formation is a highly organised process wherein single cells are recruited to form a multicellular pseudoplasmodium or slug, which will eventually form into a stalk supporting a mass of spores. Prespore cells, which occupy the rear 3/4 of the pseudoplasmodium, terminally differentiate into spores, while being lifted up by the movement and differentiation of prestalk cells. Prestalk cells are mainly found in the anterior tip of the migrating pseudoplasmodium and are divided into different sub-types based upon their expression of two genes that encode extracellular matrix proteins, the *ecmA and ecmB* genes. The slug tip contains cells that express the *ecmA* gene (pstA cells) and cells that express both the *ecmA* and *ecmB* genes (pstAB cells). The pstAB cells form an inverted cone in the center of the tip and during culmination the pstAB cells are the first cells to enter the stalk tube where they quickly differentiate into mature stalk cells. The pstA cells surround the pstB cells at the anterior; these cells transdifferentiate into pstB cells just as they enter the stalk tube[32]. A third type of prestalk cells, called pstO, express the *ecmA* gene weakly and these cells are found at the junction with the prespore region and interspersed with the prespore cells at the posterior[14]. The pstO cells are most likely identical to an earlier recognized population, the anterior-like cells (ALC), which display the characteristics of prestalk cells, while residing in the prespore region[2,72]. Some of the ALCs also express the *ecmB* gene at low levels[32]. Formation of the fruiting body is the net result of cell differentiation and an intricate system of coordinated cell movements. Both are under control of intercellular communication mediated by diffusible signalling molecules; the stalk-inducing factor DIF and its two antagonists ammonia and cyclic 3',5'-adenosine monophosphate (cAMP). cAMP plays a major role as regulator of almost all classes of developmental gene expression, chemoattractant, and intracellular intermediate for gene induction. We here review its dual role, as an inter- and intracellular signal during *Dictyostelium* development.

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Extracellular cAMP

cAMP as chemoattractant

Upon starvation *Dictyostelium discoideum* cells migrate chemotactically to form a multicellular aggregate. Soon after food deprivation, an oscillatory cAMP secretion system is induced, which generates waves of chemoattractant in the aggregative field and directs cells to move towards the oscillating centers. Streams of adhering cells form, which recruit cells from the periphery. Oscillatory signaling is made possible by the interaction of the following components: (i) an adenylyl cyclase, which produces cAMP when activated by a stimulatory G-protein and which is turned off when interacting with an inhibitory G-protein[70,74], (ii) cell surface cAMP receptors which upon occupation with cAMP firstly activate the stimulatory G-protein and later the inhibitory G-protein, causing adaptation of the response[31,59], (iii) a mechanism for cAMP secretion that has not yet been determined, (iv) an extracellular cAMP-phosphodiesterase which degrades cAMP allowing cells to return to an excitable state.

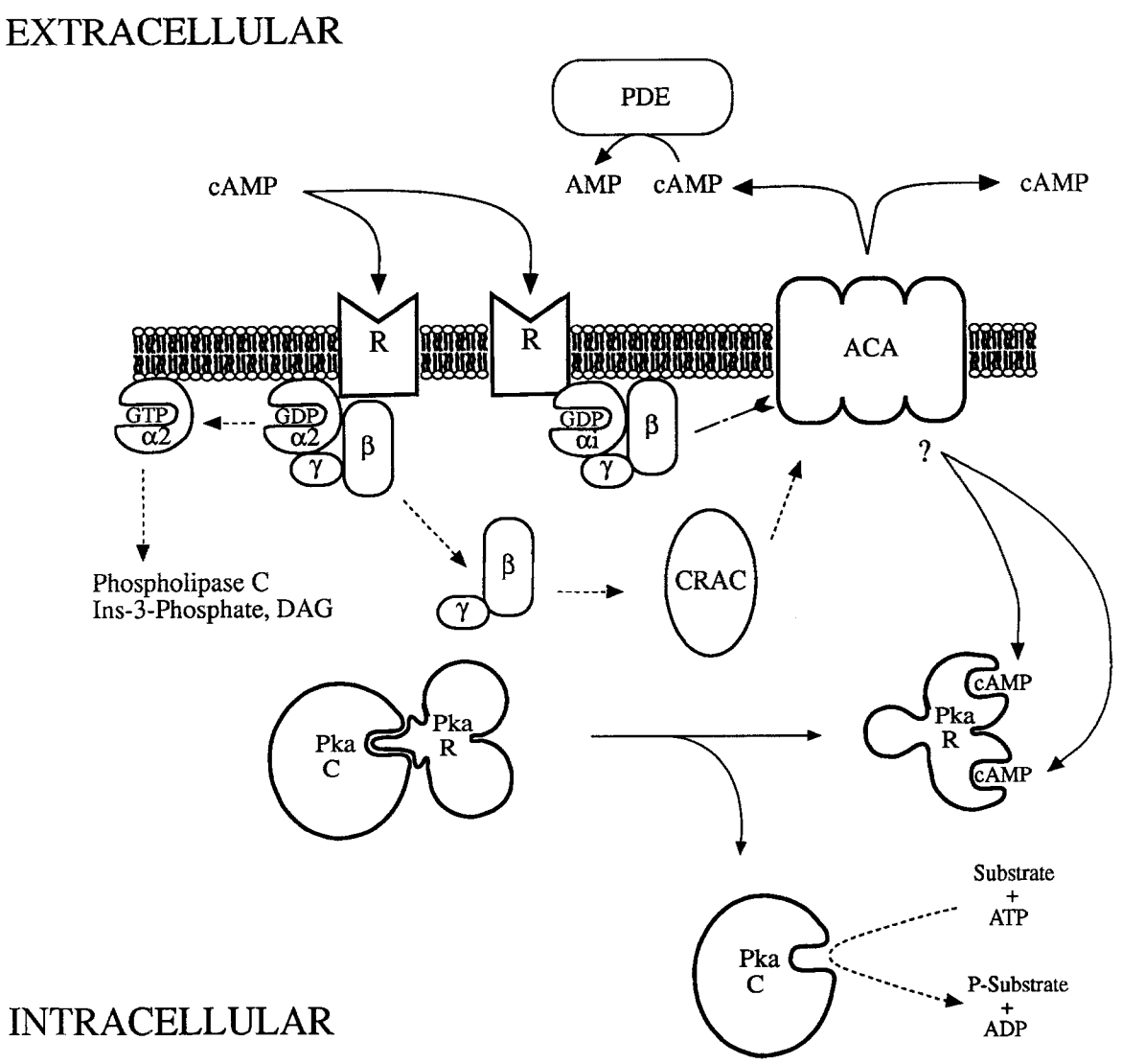

Figure 1. Extracellular cAMP binds to specific receptors (R) which are coupled to G-proteins which either stimulate via CRAC or inhibit adenylyl cyclase (ACA). The G-proteins are composed of alpha, beta and gamma subunits ($\alpha 2$ and αi respectively, β and γ). The level of extracellular cAMP is lowered by a phosphodiesterase (PDE). Intracellular cAMP binds to the regulatory subunit (R) of the cAMP dependent protein kinase (Pka) liberating the catalytic subunit (C). The source of intracellular cAMP is not yet fully understood. DAG, diacylglycerol. GTP, guanosine triphosphate, GDP, guanosine diphosphate. cAMP 3′,5′-cyclic adenosine monophosphate. Plain arrows indicate binding or dissociation. Dotted arrows indicate direct or indirect activation. Arrow barbed end indicates inhibition.

Theoretical studies have shown that such a set of components is sufficient to autonomously generate and relay cAMP oscillations[49,73]. In the following paragraphs we will review the known molecular components of this signaling system in *Dictyostelium discoideum* (see fig. 1).

Adenylyl cyclase

Extracellular cAMP is synthesized by a cellular adenylyl cyclase. Thus far two adenylyl cyclase genes have been cloned, encoding quite different enzymes. The *aca* gene, encoding a protein resembling mammalian adenylyl cyclases, is expressed at a high level during aggregation and at a reduced level during multicellular development (fig. 2). ACA is thus a likely candidate for the synthesis of extracellular cAMP during aggregation. Furthermore, its amino acid sequence shows twelve transmembrane domains suggesting an association with the plasmamembrane. The second gene (*acg*) is expressed in mature fruiting bodies and during spore germination (fig. 2). It contains a single potential transmembrane domain, and thus resembles membrane bound guanylate cyclases. Its specificity is, however, clearly directed towards cAMP[56].

ACA plays an essential role during aggregation since null (*aca*$^-$) mutants are unable to form multicellular structures. Exogenous cAMP can restore gene expression and postaggregative development of *aca*$^-$ cells when applied first in the form of nanomolar pulses and then at higher concentrations[56], indicating that the mu-

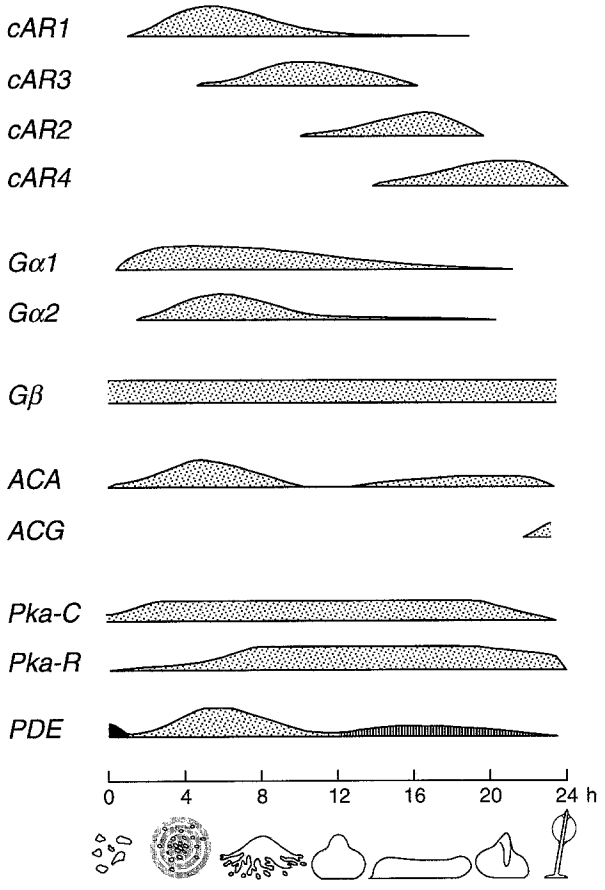

Figure 2. Expression of the mRNAs from the genes described in the text during *Dictyostelium* development. Morphological stages are referred to the approximate time of appearance at the bottom of the figure. The height of the dotted surfaces indicate approximative levels of mRNAs in relative values. For PDE the changes in shading indicate changes of transcripts due to the use of alternative promoters.

tant cells have kept their ability to respond to extracellular cAMP.

ACA is regulated through cAMP receptors (cARs) and G-proteins; at present genes for four different cARs and eight different G-proteins have been isolated[23,33,39,41,44,60,64,86]. Recent evidence from molecular genetic studies indicate that both activation and adaptation of ACA are induced upon binding of cAMP to cAR1[31,59], which is predominantly expressed shortly before and during aggregation (fig. 2). Activation is most likely mediated by the beta-gamma subunits of the heterotrimeric G-protein G_2, via a cytosolic factor termed CRAC (cytosolic regulator of adenylylcyclase). Recent studies suggest that beta-gamma subunits serve to translocate CRAC from the cytosol to the plasmamembrane where it can activate ACA[31,42,43].

One issue which remains unclear is how the specificity of transduction is achieved. There are at least eight different alpha genes, but only a single beta subunit gene in *Dictyostelium*. Other signals, e.g. folate, which also use a G-protein mediated transduction, result in totally different intracellular effects[7]. Could other pathways activated in parallel, like phospholipase C activation, play also a role in the specific activation of ACA triggered by the binding of cAMP to cAR1?

Phosphodiesterase

Repeated pulses of cAMP synthesis would steadily increase the level of extracellular cAMP without a cAMP phosphodiesterase (PDE) activity degrading this compound (fig. 1). A small amount of PDE is secreted during vegetative growth, whereas two forms with high and low Kms accumulate during the aggregation phase. Both a membrane bound and a secreted form of PDE have been described[13]. In addition to the export signal sequence, extracellular PDE seems to be lacking an N-terminal peptide, which was proposed to be involved in PDE membrane localization[58]. In the presence of pulses of cAMP, the membrane-bound form accumulates, whereas continuous cAMP increases the amount of the extracellular form[19]. The importance of PDE for *Dictyostelium* development is demonstrated by the observation that mutants lacking PDE cannot aggregate[9]. Reintroducing the gene into such a mutant almost completely restores development[18], whereas overproduction in wild-type cells blocks late development[17]. From these results one can infer a role for PDE and consequently cAMP both during the early aggregation phase and during culmination. Consistent with these multiple roles the PDE gene shows a complex regulation (fig. 2). Three separate promoter elements direct transcription of a 1.9 kb mRNA during growth, of a 2.4 kb mRNA which is cAMP inducible and appears shortly after starvation, and of a 2.2 kb mRNA during postaggregative development; the latter being found in prestalk cells only[16,57]. During early aggregation, PDE activity is fine tuned by the secretion of a phosphodiesterase inhibitor PDI, which binds to the extracellular PDE, but not to the membrane-bound form[85].

cAMP receptors

Secreted cAMP is detected by cell surface cAMP receptors (cARs). Earlier kinetic studies indicated the existence of two types of cARs, the rapidly dissociating A sites, which during cAMP stimulation revert from a high into a low affinity form (AH and AL), and the slowly dissociating BS and BSS-sites, which display a reduction of dissociation rate during cAMP stimulation[79]. These different kinetically defined sites were considered to represent different conformational forms of the chemotactic receptor depending on phosphorylation and/or interaction with intracellular effectors[76].

The binding of extracellular cAMP to its receptors induces a large number of intracellular responses such as the transient activation of effector enzymes AC (ACA)[63,67], guanylyl cyclase (GC)[50,87] and phospholipase C (PLC)[15,77] as well as increased influxes of Ca^{2+}

and effluxes of H^+ and K^+ ions ([3,46,52], see review by Newell et al. in this issue, p. 1155). Pharmacological studies using cAMP analogs showed that induction of all these responses as well as chemotaxis and the induction of gene expression are mediated by cARs[54,65,66,75,78].

Multiple *Dictyostelium* genes have been isolated which encode cAMP receptors. cAR1 is expressed maximally during aggregation and it partially overlaps with expression of cAR3. cAR2 is first transcribed when tips are formed on multicellular aggregates and cAR4 is expressed during fruiting body formation (fig. 2, for review see reference 27). The role of the different cARs in specific responses is only beginning to be resolved. Unfortunately, the cAMP analog specificity profiles of the different receptors are very similar[34], so pharmacological studies have a very limited use in attributing specific responses to specific cARs. The powerful techniques of molecular genetics have been more successful.

cAR1-minus cells obtained either by antisense or by gene disruption do not aggregate and do not show activation of adenylyl cyclase, of guanylyl cyclase or of aggregative gene expression in response to nanomolar cAMP pulses. Activation of PLC is however normal. Suprisingly, micromolar cAMP stimuli followed by a persistent stimulation with micromolar cAMP not only restored the defective responses, but also allowed $cAR1^-$ cells to form normal slugs and fruiting bodies. In addition to an action on AC and GC, cAR1 mediates adaptation of PLC, aggregative gene expression and specific aspects of adaptation of adenylyl cyclase. In *car1*$^-$/*car3*$^-$ double mutants development cannot be restored by exogenous addition of cAMP. In these cells both adenylyl and guanylyl cyclase are absent. This indicates that cAR3 is partially redundant with cAR1 and can restore the stimulatory responses in $cAR1^-$ cells, but not the inhibitory responses.

The involvement of cAR1 in multiple responses is reflected by its interaction with different G-proteins (fig. 1). Activation of PLC, GC and AC are absent in cells lacking the alpha subunit of the G_2 protein. Both PLC and GC can be inhibited by GDPβS in wild type cells, further indicating their regulation by a G-protein. AC can be activated by GTPγS in vitro in $G_{\alpha 2}$- cells, indicating the possible involvement of another G_α-subunit, but activation is completely lost in cells lacking the beta subunit protein. G_2-mediated activation of adenylyl cyclase and guanylyl cyclase are both mediated by the binding of cAMP to cAR1, whereas PLC is activated by a presently unidentified cAR.

Many responses such as the activation of AC, GC and PLC, as well as Ca^{2+} influx[52], chemotaxis and aggregative gene expression are subject to desensitization mechanisms. These desensitization mechanisms are complex and occur at several levels, such as reduction of receptor affinity, downregulation and degradation of receptors[38,80,83], as well as activation of inhibitory pathways[8,71]. During desensitization, cAR1 becomes phosphorylated while showing a lower binding affinity for cAMP (AL). Remarkably the system can revert to high binding affinity (AH) within five minutes and does not seems to require a functional G_α protein. When the putative phosphorylation sites are mutated, the transition from AH to AL is lost, but adaptation of adenylyl cyclase and aggregative gene expression still occur[10]. Some classes of mutants within the intracellular domain (class III) are also unable to couple G-protein, unable to respond by increasing Ca^{2+} influx, unable to become phosphorylated, and show no lowering in ligand binding, even though they retain their ability to bind cAMP.

Adaptation of adenylyl cyclase was partially defective and adaptation of PLC is completely defective in $cAR1^-$ cells, indicating the involvement of cAR1 in both processes. Independent experiments indicate that adaptation can occur at the level of G-proteins[70]. An as yet unidentified, pertussis toxin–sensitive protein, that is possibly a RAS type monomeric G-protein, has been found to decrease adenylyl cyclase response to subsequent cAMP binding. Furthermore, the adaptation of the PLC response seems to occur via $G_{\alpha 1}$ rather than $G_{\alpha 2}$.

cAR3 mRNA is maximally abundant at the mound stage, and it therefore partially overlaps cAR1 in its expression pattern. Its role is unclear, since $cAR3^-$ mutants show normal development[33]. cAR2 differs from cAR1 in containing homopolymeric runs of histidines and arginines at its C-terminus. cAR2 is required for normal tip and fruit formation[64] since $cAR2^-$ strains are blocked at the mound stage, and overexpress prespore genes. In wild type strains cAR2 is expressed in the prestalk zone of pseudoplasmodia; in culminants expression occurs in the stalk, but not in the basal disc and lower cup cells. cAR4 is expressed during fruiting body formation, and $cAR4^-$ mutants are perturbed in final fruit formation. They show an excess of prespore cells and reduced *ecmB* expression. Some responses such as induction of prespore genes and activation of PLC are normal in all cAR gene disruptants. Furthermore, the cAMP induced Ca^{2+} ion flux can be mediated by all cARs and does not require G-proteins. These results reflect a redundancy in the function of the cAMP receptors, without excluding the existence of additional cARs. Double or perhaps even triple gene disruptants may be required to solve this issue. Thus, to conclude, cARs show extreme flexibility in their interactions with target proteins.

cAMP as first messenger for gene regulation

Extracellular cAMP has a second known major function in the control of gene expression during all stages of development. cAMP pulses in the nanomolar concentration range repress expression of growth phase

genes[26,37], while accelerating the expression of genes involved in the aggregation process, such as cAR1 and $G_{\alpha 2}$ (fig. 2)[11,21,47]. Persistent stimulation with nanomolar cAMP concentrations induces expression of early and intermediate genes like *pde*, *cp2* and *Dd rasD*[45,51,61,66,88]. Micromolar cAMP concentrations, which are considered to accumulate in multicellular structures, induce expression of genes expressed in pseudoplasmodia mainly in prespore cells[35,51,82] and repress expression of the prestalk gene *ecmB*[6,28]. These events are triggered by the binding of cAMP to its receptors rather than diffusion within the cell, since membrane-impermeable cAMP analogs are effective[55]. Furthermore the repression of aggregation genes seems absent in cAR1$^-$ cells[31].

Intracellular cAMP

An important role for intracellular cAMP is suggested by observations that cAMP dependent protein kinase (PKA) is essential for several aspects of *Dictyostelium* development. In contrast to the mammalian enzyme which consists of two regulatory (R) and two catalytic (C) subunits, the *Dictyostelium* PKA is composed of a single R and a single C-subunit[12]. Binding of cAMP to the R-subunit dissociates the holoenzyme, liberating the active C-subunit (fig. 1). The genes encoding the C- and R-subunits have been isolated. The R-subunit gene encodes a 41 kDa protein, that closely resembles RI type mammalian subunits. As expected from the fact that the holoenzyme is an RC hetero-dimer, the *Dictyostelium* R-subunit lacks an N-terminal dimerisation domain, but like its mammalian counterpart it contains two cAMP binding sites[53]. A pseudosubstrate site within the R sequence is believed to bind the C-subunit at the catalytic site, causing the inhibition of PKA activity. No protein kinase A specific inhibitor, resembling mammalian PKI, has as yet been described in *Dictyostelium*, although *Dictyostelium* PKA activity is inhibited by bovine PKI[4].

The PKA C-subunit is a protein of 73 kDa which has been shown to bind to the R-subunit[4]. Partially purified C-subunit is inhibited by both purified R-subunit and by PKI in vitro. *Pka* C is highly homologous to the mammalian catalytic subunits except for the presence of a long N-terminal domain, which is roughly the same size as the 36 kD catalytic domain. This long N-terminal domain is unusual even when compared to the yeast PKA genes (TPK1, 2 and 3) which show a few amino acids in front of the catalytic domain. The function of the *Dictyostelium* N-terminal domain is unknown, but it contains a conserved α-helix motif, which has been proposed to be juxtaposed to the catalytic core in the tertiary structure of PKA where it could have a regulatory activity[81].

A series of experiments with cell lines expressing wild type or mutated R-subunits has indicated an essential role of PKA at several stages in development. In Rm mutant forms of the R-subunit of PKA, the gene was mutated in the two cAMP binding sites so that it no longer binds cAMP. Such a change acts as a dominant negative mutation, resulting in a protein that is able to inhibit PKA even when cAMP and wild type R protein are present. When Rm was expressed under a constitutively expressed promoter, that of the actin 15 gene, cells did not aggregate[24,68]. Similarly, a *pka* C minus strain was also found to be blocked before aggregation[48]. Expression of Rm under the *ecmA* prestalk promoter, results in slugs that are unable to culminate and that cannot differentiate into mature stalk cells even when treated with an excess of the stalk-cell morphogen DIF[25]. Similarly, expression of Rm under the control of a prespore-specific promoter blocks spore maturation[29]. Further evidence for a role of PKA in spore and stalk maturation is provided by the observation that the membrane-permeable PKA agonist 8-Br-cAMP, but not cAMP itself, can induce terminal differentiation of both spore and stalk cells[36,40].

Mutants without a functional R-subunits (*rdeC*)[69] or mutants overexpressing *pka* C[5] show a rapid development and facilitated spore formation at low density (sporogenous phenotype)[35]. A further refinement in the understanding of the effect of increased PKA activity was obtained by using cell-type specific promoters. Overexpression of *pka* C under either the *ecmA* or *ecmB* prestalk promoters blocked *Dictyostelium* development. These results, besides indicating the need of stalk cell differentiation for the correct level of PKA activity in prestalk cells, also show that prestalk cell differentiation is required for the formation of spores. Overexpressing *pka* C under a prespore promoter results in precocious spore formation and leads to the formation of a mass of spores at the bottom of the stalk. These cell lines are also capable of forming spores at low cell density (sporogenous). Spore formation, in this case where PKA is overexpressed in prespore cells, does not apparently require the presence of prestalk cells.

All these data indicate that PKA is essential during aggregation and that it plays a central role in the differentiation of stalk and spore cells. However, many aspects of the role of PKA in development remain unresolved. For example, it is not at present clear which substrates are phosphorylated by PKA in *Dictyostelium*. No CREB or CREM equivalent has been isolated from *Dictyostelium* up to now. A possible target, however, is GBF, a protein that binds to G-rich sequences common to many *Dictyostelium* genes. It has recently been shown that GBF activity is greatly reduced in psA-Rm cells (Hopper et al., personal commun.), suggesting that GBF is either a direct target or that it lies at the end of a kinase cascade involving PKA.

It is furthermore not clear how PKA becomes activated. Intracellular cAMP is present throughout development with a large increase during pseudoplasmodium formation[1,5]. Most obviously, the *Dictyostelium* adenylyl cyclases might be expected to provide cAMP for PKA activation. However, ACG is expressed only during spore germination and mutants carrying an ACG gene disruption develop normally. Cells lacking ACA, though unable to aggregate autonomously, can be induced to develop into mature spores and stalk cells by stimulation with cAR agonists that cannot activate PKA. It therefore appears that ACA is either not the only upstream activator of PKA or may not be involved at all in intracellular cAMP production.

Some processes that are blocked by inactivation of PKA are also inhibited by the weak base ammonia, which is produced in considerable amounts as an end-product of protein degradation. Ammonia blocks culmination and the differentiation of stalk cells[22]. The correlation between PKA inhibition and ammonia effects is also evident from observations that a number of "slugger" mutants, are ammonia hypersensitive[20]. Some slugger mutants are defective in DIF-induced stalk cell differentiation, but this can be restored by the PKA agonist 8-Br-cAMP[30]. The most obvious explanation for these observations is that ammonia inhibits an upstream component of the PKA pathway, e.g. the production of cAMP. Both inhibitory and stimulatory effects of ammonia on cAMP production have been described[62,84]. However, more recent data indicate that ammonia inhibits the two *Dictyostelium* adenylyl cyclases, ACA and ACG, only transiently. Remarkably, weak acids, which strongly promote stalk cell differentiation, inhibit permanently cAMP synthesis by ACA and ACG (Schaap, P., Brand, R. and Van Es, S., unpublished results). This indicates that neither ACA nor ACG can be the target for ammonia, and would appear to make it unlikely that PKA activation is triggered by ammonia depletion. However it may be that intracellular cAMP is produced by a currently unknown adenylyl cyclase or, alternatively, PKA could also be activated in a cAMP independent manner. There may be overexpression of the catalytic subunit or perhaps there is a change in the interaction of its N-terminal domain with other regulatory proteins which could compete the binding of the R-subunit.

There is suggestive evidence for as yet unidentified extracellular signals activating PKA. During normal fruiting body formation the expression of spore specific genes progresses from the apex to the base, suggesting that an inductive signal is released from the prestalk region. The presence of such a signal is also suggested by the fact that mutants expressing *pka* C from a prestalk promoter form neither stalk or spore cells. A low molecular weight inducer also appears to be required for terminal stalk cell differentiation. The requirement for the inducer for spore and stalk cell differentiation can be bypassed by 8-Br-cAMP, strongly suggesting that the inducer is an upstream activator of PKA. How this signal is transduced remains however completely obscure.

Conclusion

There is no doubt as to the essential role of extracellular cAMP as a signaling molecule regulating morphogenetic movement and gene expression at almost all stages of *Dictyostelium* development. For these functions cAMP is detected by surface cAMP receptors and transduced through G-proteins coupled to target enzymes.

The requirement of PKA for several phases of development suggests that intracellular cAMP is equally important for development. Here we are faced with an apparent conundrum; that both the known *Dictyostelium* adenylyl cyclases appear to be dispensable for development, provided that the correct regime of cAMP stimuli is provided to activate cARs.

Alternative modes of PKA activation or of cAMP production can provide plausible explanations, but at this stage they lie entirely within the realms of speculation. However, it seems very likely that, given the rapid pace at which signaling pathways are presently being unraveled in *Dictyostelium*, this issue will be resolved in the near future. It also seems very likely that identification of the components of these complex signaling pathways will offer novel insights into signal transduction in higher organisms.

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